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The Effect of Certain SH-group Inhibitors on the Growth and Respiration of *Staphylococcus* Strains

Влияние некоторых ингибиторов групп SH на рост и дыхание *Staphylococcus aureus*

Wpływ niektórych inhibitorów grup SH na wzrost i oddychanie szczepów *Staphylococcus aureus*

Mercury compounds, iodoacetate and allicin — the active principle of garlic — are known inhibitors of SH-group enzymes. It has been observed that the majority of penicillinase producing strains of Staphylococcus aureus can show a marked degree of resistance to mercuric chloride, which is in close correlation with their epidemic properties (1, 4, 5, 6). As pointed out by Richmond and John (3) both characteristics, that is the ability to form penicillinase and mercury resistance are genetically linked, being incorporated in an extrachromosomal element called „penicillinase plasmid" (2, 3).

The aim of the present paper is to study whether the $HgCl_2$ — resistant staphylococcus will also show resistance to other SH-group inhibitors in comparison with the mercury sensitive organism. Chlormercuribensoate, iodoacetate, garlic juice and allicin, the active substance of the latter, were used for this purpose.

MATERIALS AND METHODS

Strains

For these experiments two strains were used: *Staphylococcus aureus* No 31, sensitive to mercuric chloride and penicillin, and *Staphylococcus aureus* No 15, resistant to both agents.

Media and cultivation of the strains

1% peptone agar (1) was used throughout the whole course of experiments, to which various amounts of inhibitors were added. The minimum inhibitory

concentratioi of chlormercuribensoate and of iodoacetate was defined on a series of peptone agar plates containing the former in the range from 1 : 10,000 to 1 : 400,000 and iodoacetate — from 1 : 5,000 to 1 : 500,000. Each plate containing an inhibitor was inoculated with a large loopful of an overnight broth culture and the growth observed after 24, 48, and 74 hours of incubation. In another series of experiments, cysteine was included in the medium together with the inhibitor.

The paper disc method and the agar dilution method were employed for testing the inhibitory effect of garlic juice. Strains were cultured overnight in broth, diluted 1 : 100 and then spread over the agar plate. Paper discs were placed on the surface of the medium and then 0,02 ml of various dilutions of freshly prepared garlic juice (undiluted, diluted 1 : 2, 1 : 3, 1 : 5, 1 : 10 and 1 : 100) were added to the disca. The plates were incubated overnight. Garlic juice was pressed out of the throughly crushed bulbs and used in experiments after 1 hour incubation at 37°.

In the agar dilution method the final dilutions of the juice were as follows: 1 : 10, 1 : 25, 1 : 50, 1 : 100, 1 : 200 and 1 : 400. Plates were prepared in duplicates. In one lot of the plates cysteine was incorporated (0.005M). All plates were inoculated with a large loopful from an overnight broth culture of both strains. The results were read after 24, 48 and 72 hours of incubation at 37°.

The effect of allicin on the growth of *Staphylococcus aureus* strains was defined by means of the paper disc method. The agar medium was flooded with an overnight broth culture diluted 1 : 100, the excess removed and then paper discs placed, to which 0.02 ml of the mixture of alliin and the enzyme (alliinase) obtained from the garlic, were added. 4.3 mg of alliin was suspended in 1.0 ml of water and mixed with 1 ml of crude enzyme preparation. The mixture was kept at room temperature and applied successively to the discs at: 0, 15, 30, 60, 120, 240, 360 and 480 min. During the incubation alliin was converted enzymatically into the active subtance — allicni. Each paper disc contained 21 μg of allicin.

The crude preparation of alliinase was obtained according to Stoll and Seebeck (7). 100 g of the fresh, frozen garlic was crushed throughly and extracted with 400 ml of water at 37°. The extract was filtered and the supernatant treated with 21 ml of 10% acetic acid. The obtained precipitate was centrifuged down, suspended in 150 ml of water and then treated with 10% ammonia to reach pH 6.4. The reaging mixture was filtered and the clear supernatant treated again with acetic to reach pH 4.0. The resulting precipitate was centrifuged down and the pellet dissolved in 400 ml of 1/15 phosphate buffer, pH 6.4. This enzyme preparation was kept in the frozen state.

Cells for manometric experiments were obtained according to the method previously described (6).

Analytical method

Oxygen uptake was measured by the conventional Warburg technique (9). Each vessel contained: 0.5 ml of cell suspension (15—20 mg wet weight), 1.5 ml of phosphate buffer 0.1 M, pH 7.0, 0.2 ml of 0.1 M glucose and in the two side arms: 0.5 ml volumes of appropriate concentrations of inhibitors and cysteine, respecively. The center well contained filter paper soaked with 0.2 ml of 20% KOH. Chlormercuribensoate was used to reach the final concentrations: 7.2×

$\times 10^{-6}$ M, 3.6×10^{-5} M and 7.2×10^{-5} M. Iidoacetate was used at concentrations: 2×10^{-5} M, 10^{-4} M, 10^{-3} M and 10^{-2} M. Garlic juice was used in following amounts: 0.5 ml and 1.0 ml of freshly prepared juice. In another experiment allicin, obtained from pure alliin, was used at concentrations: 10^{-2} M and 10^{-3} M.

Chemicals

Alliin was kindly supplied by Sandoz, Schwitzerland. Chlormercuribensoate and iodoacetate were the products of DDH, England. Cther chemicals were reagent grade products of FOCH, Gliwice, Poland.

RESULTS

Table 1

Table 1 indicates that the M.I.C. (minimum inhibitory concentration) of chloromercuribensoate for strain 31 was 1 : 50.000, while for strain 15 — 1 : 10.000. As regards to iodoacetate, there was no diference in the sensitivity of both strains in the growth test. M.I.C. for strain 15 and 31 was the same — 1 : 10.000. Both inhibitors had no inactivating effects on the growth of either strain in the presence of 0.005 M of cysteine.

Table 1. Minimum inhibitory concentration of chlormercuribensoate and iodoacetate for the growth of *Staphylococcus aureus* 15 and 31

Concentration of chlormercuribensoate		Strain		Concentration of iodoacetate		Strain	
M	Dilution w/v	15	31	M	Dilution w/v	15	31
2.64×10^{-4}	1:10,000	—	—	1.08×10^{-3}	1:5,000	—	—
1.06×10^{-4}	1:25,000	+	—	5.40×10^{-4}	1:10,000	—	—
5.30×10^{-5}	1:50,000	+	—	2.70×10^{-4}	1:20,000	+	+
2.64×10^{-5}	1:100,000	+	+	1.80×10^{-4}	1:30,000	+	+
1.32×10^{-5}	1:200,000	+	+	1.08×10^{-4}	1:50,000	+	+
6.60×10^{-6}	1:400,000	+	+	1.08×10^{-5}	1:500,000	+	+

Table 2

Table 2 shows results of the inhibitory effect of garlic juice and of allicin on the growth of staphylococci in the paper disc method. The sensitivity of strain 15 and 31 in the growth test was identical.

Table 2. The inhibitory effect of garlic juice and allicin on the growth of *Staphylococcus aureus* strains 15 and 31 in the paper disc method

Concentration of garlic juice and of allicin	Zone of inhibition (mm)	
	Strain 15	Strain 31
undiluted	35	35
diluted 1:2	28	27
„ 1:3	22	23
„ 1:5	21	21
„ 1:10	0	0
„ 1:100	0	0
allicin — 21 μg in a disc	40	40

Also in the agar dilution method 1 : 200 dilution of the juice was inhibitory for buth strains after 24 hours of incubation (table 3).

Table 3

After further 24 hours at 37° both strains grew luxuriantly on plates containing inhibitor diluted 1 : 200, while dilution 1 : 100 was inhibitory for both strains even after 5 days of incubation at 37°. Cysteine (0.005 M) included in the medium protected both strains from the inhibitory action of garlic.

The effect of inhibitors on glucose oxidation.

Table 3. The inhibitory effect of garlic juice on the growth of *Staphylococcus aureus* strains 15 and 31 in the agar dilution method

Strain No	Plates observed after hours	Dilutions of garlic juice in agar				
		1:10	1:50	1:100	1:200	1:400
15	24	—	—	—	—	+
	48	—	—	—	+	+
	72	—	—	—	+	+
31	24	—	—	—	—	+
	48	—	—	—	+	+
	72	—	—	—	+	+

+ = confluent, growth, — = no growth.

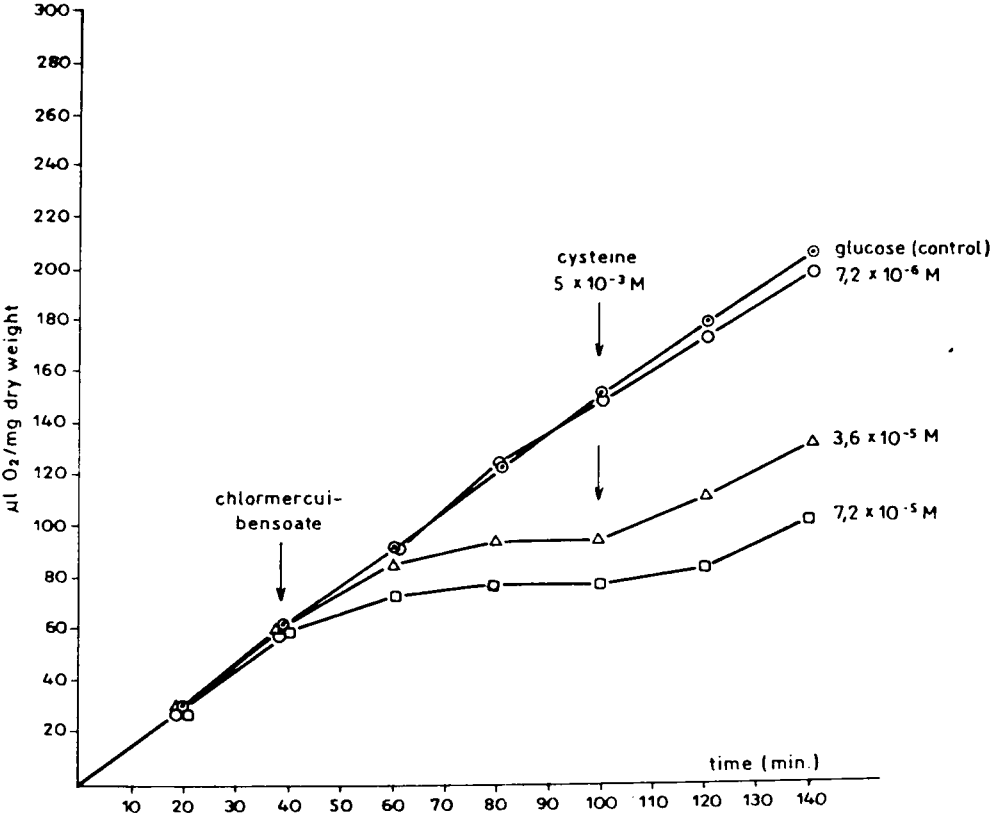

Fig. 1a. The effect of various cencentrations of chlormercuribensoate on the oxidation of glucose by *Staphylococcus aureus* strain 15

Fig. 1a and 1b show the effect of various concentrations of chlormercuribensoate on the oxygen uptake of strain 15 and 31, with glucose as a substrate. No difference in sensitivity could be observed between the two strains studied. The rate of inhibition of respiratory activity in the presence of the inhibitor was similar for both strains, although in the growth test strain 15 was more resistant than strain 31. Cysteine (0.005 M) reactivated the respiration of both strains to the same extent.

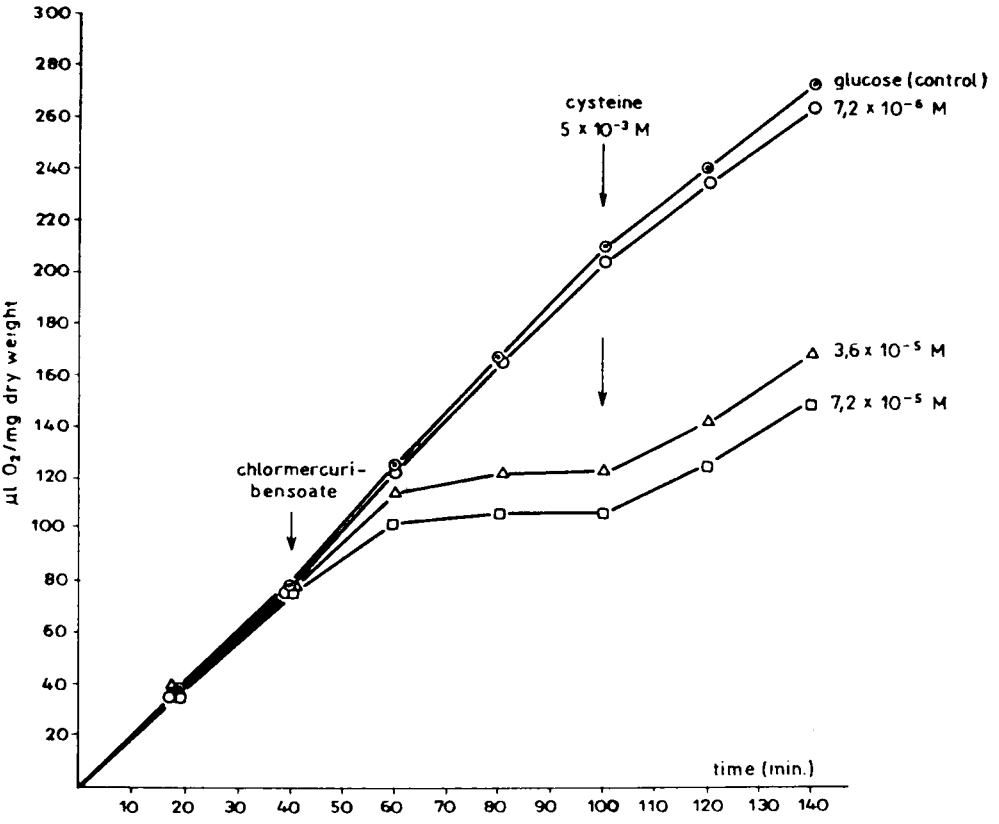

Fig. 1b. The effect of various concentrations of chlormercuribensoate on the glucose oxidation by *Staphylococcus aureus* strain 31

As regards iodoacetate (Fig. 2a and 2b), the glucose consumption by strain 15 and 31 was inhibited to the same degree, the same was true in the growth test. The addition of cysteine was not markedly effective on the reactivation of respiration of staphylococci.

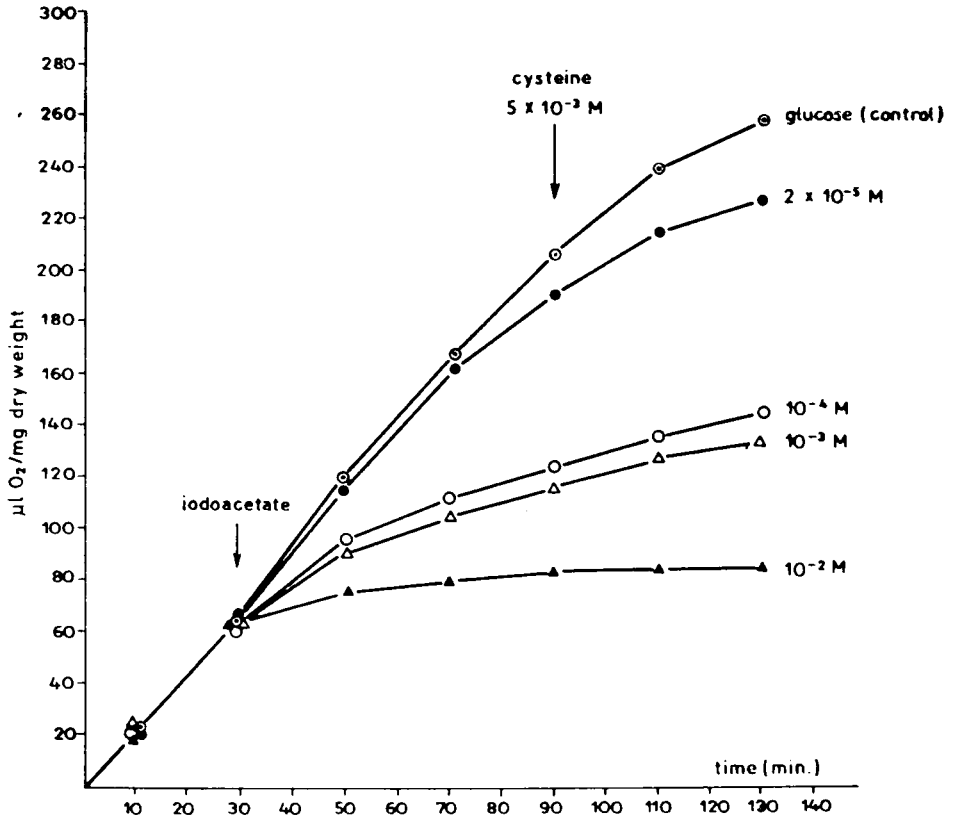

Fig. 2a. The effect of various concentrations of jodoacetate on the glucose oxidation by *Staphylococcus aureus* strain 15

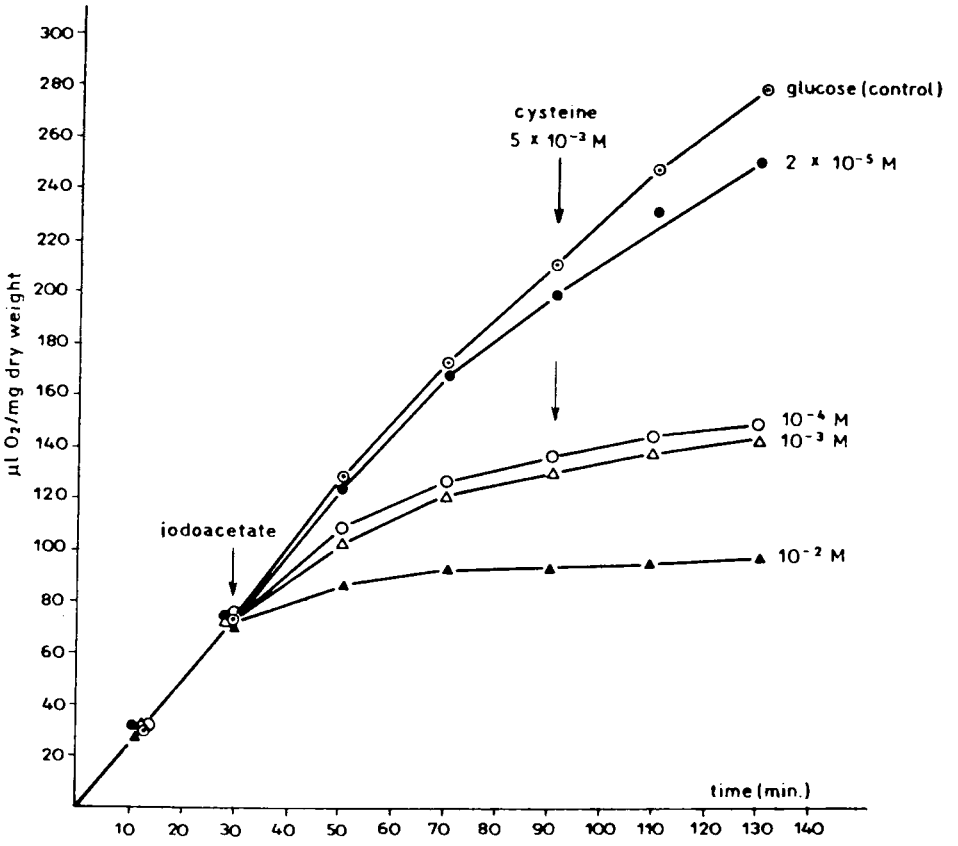

Fig. 2b. The effect of various concentration of jodoacetate on the glucose oxidation by *Staphylococcus aureus* strain 31

Fig. 3 and Fig. 4 show that allicin at concentrations 10^{-3} M and 10^{-2} M did not have any significant effect on the glucose oxidation by both strains. The same was observed with the garlic juice in Warburg experiment, while in the growth test both agents were strongly inhibitory for both strains.

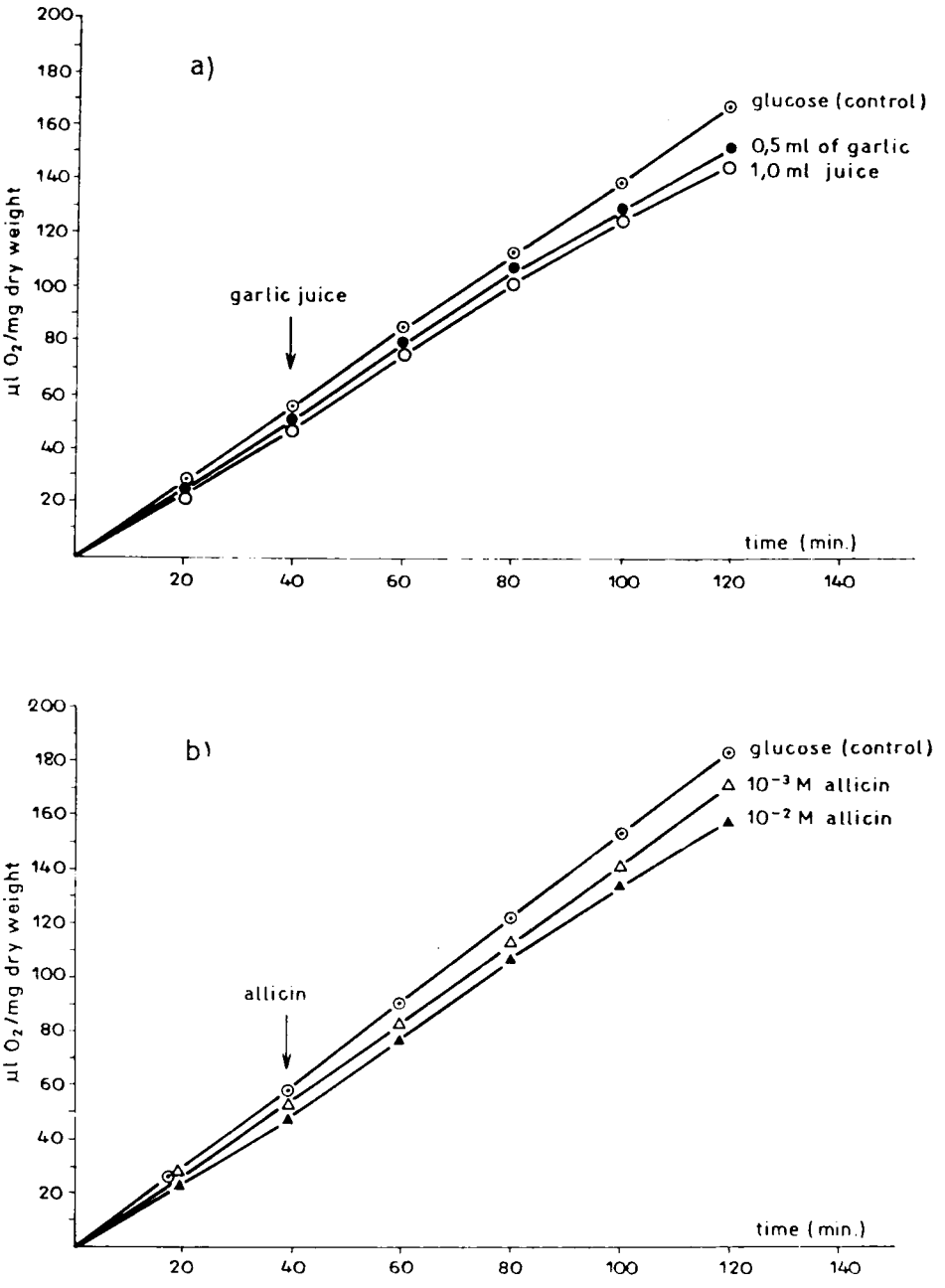

Fig. 3. The effect of garlic juice — a) and allicin, — b) on the glucose oxidation by *Staphylococcus aureus* strain 15

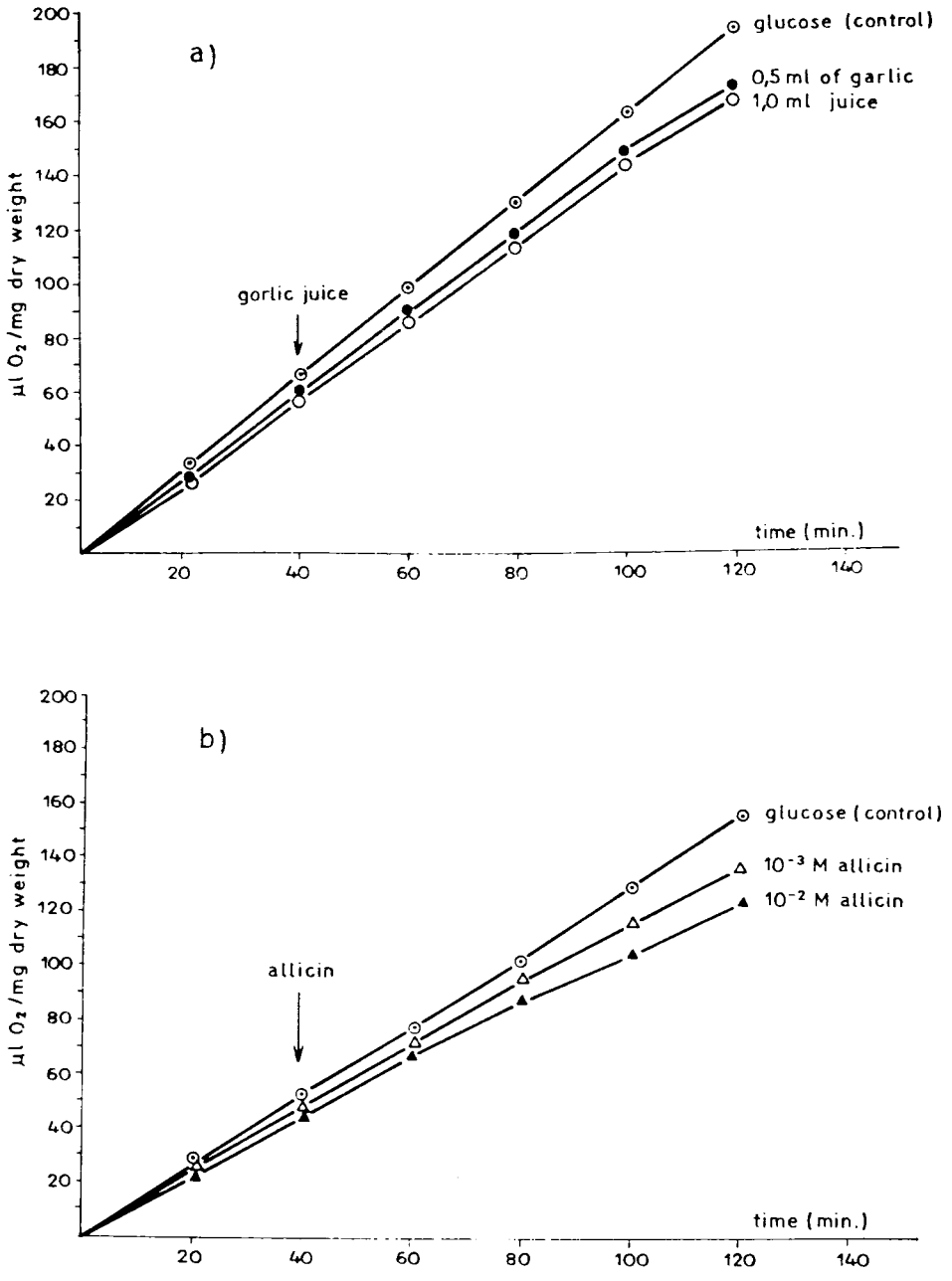

Fig. 4. The effect of garlic juice — a) and allicin, — b) on the glucose oxidation by *Staphylococcus aureus* strain 31

DISCUSSION

The aim of the present paper was to study whether $HgCl_2$ resistant *Staphylococcus aureus* strain, possessing penicillinase plasmids, will also show resistance to other SH-group inhibitor, in comparison with the mercury sensitive. organism. Staphylococcal resistance to mercuric chloride has been accepted as the criterion of their epidemic properties (1, 4). Genetic studies have revealed that resis'ance to mercury and to other inorganic ions, and also the ability to form penicillinase plasmid (2, 3). The nature of resistance to those ions remains obscure. Previous work (6) has showed that the mercury resistant strain 15 oxidised glucose at normal rate in the presence of such concentrations of $HgCl_2$, which markedly inhibited respiration of strain 31. As regards chlormercuribensoate, strain 15 was more resistant, but only in the growth test. M.I.C. for strain 15 was 1 : 10.000, while for strain 31 — 1 : 50.000. In Warburg experiments both strains behaved equally.

Iodoacetate was inhibitory for both strains to the same degree in the growth test and in Warburg experiments.

Quite unexpected results were obtained with the garlic juice and allicin in Warburg experiments. The inhability of them to suppress glucose oxidation by staphylococci seems rather puzzling, since it is known, that allicin, the active substance of garlic is an inhibitor of respiratory pathway enzymes, possessing SH-groups (8, 10). As stated by M. S z y m o n a (8), such fungi as *Candida albicans* and *Trichophyton cerebriforme* were very sensitive to garlic, both in growth test and in the Warburg experiment; 5 drops of fresh juice inhibited almost completely glucose oxidation by *Candida albicans*, while in our tests even 50 drops (1.0 ml) did not cause similar effect. This seems to indicate that in *Staphylococcus aureus* strain SH-enzymes are protected from the action of garlic in some unkown way and that fungi lack such protection mechanism. This problem is being further investigated by one of us (Z. T.). Preliminary observations (unpublished) have revealed that garlic juice in the amount of 0.1 ml or 0.2 ml stimulated endogenous respiration of *Stypholococcus aureus* Oxford even to a greater extent than glucose, while endogenous respiration of *Candida albicans* was rather inhibited by that amount of the juice. The same difference was obtained when glucose was used as a substrate, although in the growth tests both organisms were equally sensitive. This may suggest that during respiration in buffer staphylococci are protected from the inhibitory action of garlic, at least during the time of observation, and that inhibilition takes place only when growth occurrs. The fact that the addition of garlic juice to the growth medium together with cysteine

enabled staphylococci to grow, confirms the view that garlic inhibits the growth of that microorganism by combining with its SH-group enzymes (8, 10).

In conclusion, the presented results indicate that the mercury--resistant and -sensitive staphylococci do not show any difference in sensitivity to other SH-group inhibitors, except that strain 15 was 5 times more resistant to chlormercuribensoate in the growth test, as compared with strain 31.

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STRESZCZENIE

Założeniem pracy było zbadanie, czy szczep *Staphylococcus aureus*, oporny na chlorek rtęciowy, będzie wykazywał oporność również na inne inhibitory grup SH w porównaniu ze szczepem wrażliwym. Stosowano chlororteciobenzoesan, jodooctan, sok czosnkowy oraz allicynę — aktywną substancję czosnku. Hamujące działanie powyższych inhibitorów na wzrost badanych szczepów określano metodą rozcieńczeniową w agarze i metodą krążków bibułowych. Wpływ inhibitorów na oddychanie badano w aparacie Warburga w obecności glukozy jako substratu.

Nie wykazano różnic we wrażliwości szczepów na chlororteciobenzoesan i jodooctan w doświadczeniach manometrycznych, jedynie szczep oporny na $HgCl_2$ był pięciokrotnie bardziej oporny na organiczny związek rtęci w próbie wzrostowej, w porównaniu ze szczepem wrażliwym.

Czosnek i allicyna wykazywały silne właściwości przeciwgronkowcowe w próbie wzrostowej, natomiast nie miały istotnego działania hamującego na oddychanie szczepów w obecności glukozy. Obserwacja

ta jest interesująca z uwagi na to, że allicyna jest inhibitorem enzymów oddechowych posiadających grupy SH, co potwierdza osłaniające działanie cysteiny na wzrost badanych szczepów. Wydaje się prawdopodobne, że gronkowce posiadają jakiś mechanizm osłaniający ich grupy SH, który, jak wykazali inni autorzy, nie występuje u *Candida albicans*, drobnoustroju bardzo wrażliwego zarówno w próbach wzrostowych, jak i w doświadczeniach manometrycznych.

PODPIS POD RYCINY

Ryc. 1a. Wpływ różnych stężeń chlororterciobenzoesanu na uttlenianie glukozy przez szczep *Staphylococcus aureus* 15.

Ryc. 1b. Wpływ różnych stężeń chlororterciobenzoesanu na utlenianie glukozy przez szczep *Staphylococcus aureus* 31.

Ryc. 2a. Wpływ różnych stężeń jodooctanu na utlenianie glukozy przez szczep *Staphylococcus aureus* 15.

Ryc. 2b. Wpływ różnych stężeń jodoooctanu na utlenianie glukozy przez szczep *Staphylococcus aureus* 31.

Ryc. 3. Wpływ soku z czosnku a) i allicyny b) na utlenianie glukozy przez szczep *Staphylococcus aureus* 15.

Ryc. 4. Wpływ soku z czosnku a) i allicyny b) na utlenianie glukozy przez szczep *Staphylococcus aureus* 31.

TYTUŁY TABEL

Tab. 1. Minimalne stężenie chlororterciobenzoesanu i jodooctanu hamującego wzrost *Staphylococcus aureus* 15 i 31.

Tab. 2. Hamujący wpływ soku z czosnku i allicyny na wzrost szczepów *Staphylococcus aureus* 15 i 31 w metodzie krążków bibułowych.

Tab. 3. Hamujący wpływ soku z czosnku i allicyny na wzrost szczepów *Staphylococcus aureus* 15 i 31 w metodzie rozcieńczeniowej w agarze.

РЕЗЮМЕ

Исследовалась сопротивляемость штамма *Staphylococcus aureus*, устойчивость которого к хлорной ртути известна, к другим ингибиторам групп SH по сравнению с чувствительным штаммом. Применялись хлормеркурийбензоат, монойодоацетат, чесночный сок и аллицин — активное вещество чеснока. Тормозящее действие вышеназванных ингибиторов на рост штаммов определялось методом разбавления в агар-агаре и методом бумажных кружков. Влияние ингибиторов на дыхание исследовалось в аппарате Варбурга, где в качестве субстрата присутствовала глюкоза.

Не обнаружено разниц в устойчивости штаммов к хлормеркурийбензоату и монойодоацетату в манометрических исследованиях, только штамм, устойчивый к $HgCl_2$, был в пять раз больше устойчив к ор-

ганическому соединению ртути в возрастном тесте по сравнению с чувствительным штаммом.

Чеснок и аллицин обнаруживали сильные антистафилококковые свойства в возрастном тесте, в то же время не оказывали существенного тормозящего действия на дыхание штаммов в присутствии глюкозы. Эти наблюдения представляют интерес по той причине, что аллицин является ингибитором дыхательных ферментов, содержащих группы SH, что подтверждает защитное действие цистеина на рост исследуемых штаммов. Возможно, что у стафилококков есть какой-то механизм, защищающий их группы SH, который (как показали другие исследователи) не наблюдается у *Candida albicans*, микроорганизме, очень чувствительным как в возрастных тестах, так и в манометрических исследованиях.